Determination of the Methyl Ester Content of Pectin

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Sources of error in the Zeisel and saponification methods for determining the methyl ester content of pectin have been discovered and improved procedures are presented. A third method, utilizing the specific action of pectase for hydrolyzing pectin methyl ester, is recommended for general use as more rapid than the Zeisel method and involving fewer sources of error than the saponification method.

THE methyl ester content of pectin and its determination have assumed increasing importance in pectin chemistry, especially since the development of calcium pectinate gels (*1, 2, 8, 12*) from low-ester pectins. [Although the term "methoxyl" has been used for many years in the literature on pectin, it is erroneous as applied to pectin. Pectin contains methyl ester groups but probably no methoxyls. The term "methyl ester" is used in this paper, all analyses being calculated as methoxyl (% CH_3O).] Such properties as the viscosity of pectinate solutions (*12*), the setting time of pectin jellies (*1, 12*), the formation and stability of calcium pectinate gels (*1, 8*), and the solubility of pectin (*17*) are greatly influenced by the degree of esterification. Consequently, accurate methods for determining the methyl ester content of pectin are essential.

The Zeisel and saponification methods are the two principal procedures now used for determination of the methyl ester content of pectin. The saponification method is empirical, although it is frequently not recognized as such in the literature. It is considered accurate because it usually gives results in agreement with those by the Zeisel method, which is the reference method.

Both the Zeisel and saponification methods have given erratic results in the authors' hands. Sources of error in these methods have been discovered, and improved procedures for both methods have been developed. A third method, using pectase for estimation of the methyl ester content of pectin, has certain advantages over the others.

ZEISEL METHOD

The principal cause for the erratic Zeisel results was discovered by Jansen, Waisbrot, and Rietz (*11*), and independently by the authors. It was found that a portion of the ethanol commonly used in the preparation of pectin is so strongly held that it cannot be removed by drying in vacuo at 80° C. Ethanol forms ethyl iodide which, being volatile, is measured and calculated as methoxyl in the usual Zeisel procedure. Since the amount of adsorbed ethanol may be as much as 4% of the pectin by weight, the error from this source may be appreciable.

Jansen *et al.* detected the presence of bound ethanol in pectin by the fact that it was possible to lower the Zeisel value by subjecting the pectin to a water-vapor treatment. Ethanol was considered to be completely removed when continued water-vapor treatment no longer reduced the Zeisel value, and the Zeisel value corresponded to the saponification value. Obviously this proof is indirect and does not preclude the possibility that a portion of the ethanol is not removed or that the saponification values are in error. Data presented in this paper show that results by the saponification method depend upon the conditions of saponification and the type of pectin being analyzed. Therefore, the saponification method cannot be considered a reference method.

The studies herein described offer a more direct proof of the presence of adsorbed ethanol and completeness of its removal—namely, by the separation and measurement of ethyl iodide in the Zeisel distillate. The Zeisel method as modified by Clark (*3*) was used throughout this study, except that an aqueous suspension of red phosphorus was used in the washer.

A sample of apple pectin which had been precipitated by ethanol and then dried for 24 hours at 60° C. in an air oven was analyzed by the Zeisel procedure and gave a value of 12.0% methoxyl (m.f.b.). When the analysis was repeated and the volatile iodides were subjected to the trimethylamine separation method (*4*), the sample analyzed 9.00% methoxyl and 4.5% ethoxyl (or 3.1% calculated as methoxyl).

Attempts to remove adsorbed ethanol from the pectin by drying in vacuo for 2 hours at 80° C. were unsuccessful. The ethanol was removed, however, by substitution with water vapor in the following manner:

A sample of the same ethanol-precipitated apple pectin was placed in the inner chamber of a Conway diffusion cell with distilled water in the outer ring. The cell was placed under a bell jar. The jar was evacuated and sealed off, and the sample allowed to stand overnight. The sample was then dried for 2 hours in vacuo at 80° C. The methoxyl value, as determined by the Zeisel method, was 9.44%. Repetition of the water-vapor treatment again yielded a methoxyl value of 9.44%.

If the per cent methoxyl from the trimethylamine separation (9.00%) is calculated to the alcohol-free basis, a value of 9.43% is obtained. The close agreement between this result and that obtained on the vapor-treated sample (9.44%), and the fact that a second water-vapor treatment did not change the methoxyl value, show that a single vapor treatment removed all the adsorbed ethanol. These facts also indicate that the vapor-treated sample contained no groups other than methoxyl capable of forming volatile iodides. Vapor-treating the pectin samples at reduced pressure increases the rate of ethanol-water exchange considerably. Jansen, Waisbrot, and Rietz (*11*) used a vapor treatment of 2 days at atmospheric pressure.

Table I shows the methoxyl values of a number of pectins before and after vapor treatment. It is apparent from these data that failure to remove ethanol from the dried sample may cause the result to be almost 3% too high. The discrepancy was much less for acid-deesterified than for enzyme-deesterified pectins.

Many experimentally and commercially prepared pectins are precipitated by acetone, ethanol, or isopropanol. The alcohols react with hydriodic acid to form volatile iodides, and under certain conditions acetone may likewise yield a volatile iodide. Therefore, the procedure for determining the methoxyl values of pectins by the Zeisel method should include a preliminary water-vapor treatment of the sample unless it is definitely known that the pectin contains no adsorbed solvent capable of interfering with the analysis. Jansen, Waisbrot, and Rietz (*11*) have also shown that acetone does not interfere with the Zeisel analysis and that isopropanol may be removed by an Abderhalden dryer.

It might appear that a satisfactory method for eliminating interfering solvent from pectin prior to Zeisel analysis would be

Table I. Methoxyl Values of Pectins Determined by Zeisel Method

(Before and after vapor treatment to remove absorbed ethanol)

Sample No.	Type of Demethylation	Per Cent CH_3O[a] Before vapor treatment	After vapor treatment
91	None	12.0	9.4
91B	Acid	7.9	6.8
91C	Acid	7.1	5.1
91E	Acid	1.9	1.7
91G	Enzyme	8.4	6.0
91J	Enzyme	4.3	2.0

[a] Moisture- and ash-free basis.

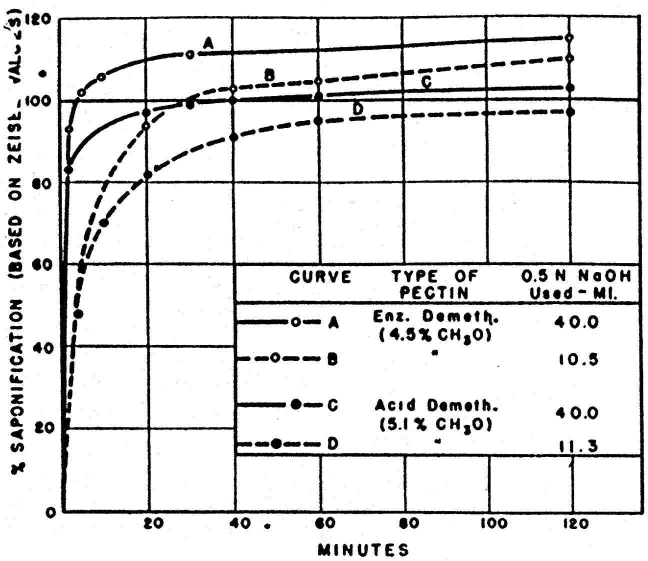

Figure 1. Rate of Saponification of Pectin

Saponification of 2 grams of pectin in 400 ml. of water at 25° C. Amount of alkali used in B and D was 5 ml. in excess of amount equivalent to CH_3O values of respective pectins

to dissolve it in water and precipitate it with a noninterfering solvent, such as acetone. However, this procedure would also remove a portion of the nongalacturonide constituents and thus alter the original methyl ester content of the pectin.

Since the error in the Zeisel analysis caused by adsorbed solvent was reported only recently, it is probable that many of the pectin methoxyl values recorded in the literature are erroneous.

SAPONIFICATION METHODS

Saponification of pectin by dilute alkali (*6*) has been used by various investigators (*5, 9, 14, 15, 16*) as a quantitative method for the estimation of pectin methyl ester. It is more rapid than the Zeisel method and requires no special apparatus. It is an empirical procedure, however, and the results depend upon the choice of conditions. Since the Zeisel values for pectin reported in the literature are probably in error, it follows that the saponification method, which is based upon these Zeisel values, is likewise probably in error. In view of the new Zeisel values, experimental conditions for the saponification method must be reinvestigated.

The usual procedure for saponification, as described by Myers and Baker (*14*), is to add 20 ml. of 0.5 *N* sodium hydroxide to a neutralized pectin solution containing 1 gram of pectin dissolved in 200 ml. of water, and saponify at room temperature for 2 hours. Methoxyl values obtained by this procedure are recorded in Table II. Comparison of the data shows that this method gives higher values than the Zeisel method. The difference is much less for acid-deesterified than for other types of pectins.

To work out procedures suitable for different kinds of pectin, it was necessary to ascertain the rate and extent of saponification under various conditions of time, temperature, and alkali concentration.

The rate of saponification was studied with an enzyme-deesterified pectin (4.51% CH_3O) and an acid-deesterified pectin (5.13% CH_3O), both purified by the method of Olsen *et al.* (*15*). These two samples were chosen because they have comparable methyl ester contents and yet represent two types of pectins that differ in saponification behavior.

Two grams of pectin pretreated to remove adsorbed ethanol were weighed into a 600-ml. beaker, moistened with ethanol, and dissolved with 400 ml. of distilled water using a mechanical stirrer. The solution was titrated to pH 7.5 with 0.500 *N* sodium hydroxide, a Beckman pH meter being used to determine the end point. [Hinton (*9*) found the neutralization point of pectinic acids to be pH 7.5. Although the neutralization point varies slightly with the concentration of pectin, the authors found that the value 7.5 was a satisfactory end point for the range of pectin concentrations encountered in the present study.] The neutralized solution was transferred to a stoppered flask, adjusted to 25° C., and saponified by adding a measured volume of 0.500 *N* sodium hydroxide. When the saponification had proceeded for the desired time, 0.500 *N* sulfuric acid was added in an amount equal to the sodium hydroxide used for the saponification. The solution was then titrated to pH 7.5 as in the first titration, 1 ml. of 0.500 *N* sodium hydroxide used in the final titration being equivalent to 0.0155 gram of methoxyl. Two amounts of 0.500 *N* sodium hydroxide were used—40 ml. per 2 grams of pectin, corresponding to the Myers-Baker saponification procedure, and a smaller amount, which was 5 ml. in excess of the amount equivalent to the methoxyl values of the respective pectins.

It is apparent from a study of the data in Figure 1 that no single set of conditions can be employed for the accurate determination of methyl ester in various types of pectin by saponification. Additional studies were made, in which temperatures as low as 0° C. and as high as 40° C. were used. Other alkaline reagents, such as alkaline salts and organic bases, were also used, but no better results were obtained. A series of carbonate buffers in the range of pH 9.9 to 11.0 was tried without success. In all cases it was impossible to reduce the large saponification error that occurred when enzyme-demethylated pectin was saponified under conditions necessary to saponify the acid-deesterified pectin completely.

Recent studies in this laboratory (*7*) have shown that acid deesterification progressively removes the pectin nongalacturonide material from the polygalacturonide portion, whereas enzyme deesterification does not appreciably affect these nongalacturonide materials. It is apparent that the methoxyl values of pectins differing in galacturonide content are not strictly comparable. A more accurate basis for comparison—namely, the per cent esterification of the galacturonide chain—has been presented (*7*).

The acid-deesterified pectin used in this study contained only 16.9% of nongalacturonide material, little if any of which was araban. The enzyme-deesterified pectin, which had approximately the same nongalacturonide content as the pectin from which it was derived (24.5%), contained both araban and galactan. As pointed out by Hirst and Jones (*10*), acid removes the araban fraction much more readily than the galactan fraction. Thus one may conclude that the araban fraction, or some other easily removable constituent, is responsible for the observed differences in the saponification behavior of these two

Table II. Methoxyl Values of Pectins as Determined by Different Methods

(All values calculated on moisture-, ash-, and ethanol-free basis)

Sample No.	Description	Zeisel Method	Saponification Methods: Myers-Baker	Method A	Method B	Pectase Method
		%	%	%	%	%
123	Commercial citrus pectin	10.04	10.87	9.60	..	9.80
102	Commercial apple pectin	7.05	7.58	6.56	..	6.99
115	Purified apple pectin	10.24	10.59	10.06	..	10.17
91	Purified apple pectin	9.44	10.09	9.34	..	9.39
91B	Acid-demethylated apple pectin	6.76	6.73	...	6.64	6.55
91C	Acid-demethylated apple pectin	5.13	5.25	...	5.12	5.04
91E	Acid-demethylated apple pectin	1.74	1.98	...	1.91	1.79
91G	Enzyme-demethylated apple pectin	6.01	6.62	5.84	..	5.99
91H	Enzyme-demethylated apple pectin	4.51	5.24	4.43	..	4.57
91J	Enzyme-demethylated apple pectin	1.99	2.60	1.92	..	2.02
115B	Alkali-demethylated apple pectin	5.66	6.53	5.62	..	5.73
	Average deviation from Zeisel	Std.	0.50	0.21	0.10	0.09
	Precision of method	±0.04	±0.03	±0.03	±0.02	±0.02

pectins. Nondeesterified and alkali-deesterified pectins show the same type of saponification behavior as enzyme-deesterified pectin.

Since no single set of conditions could be used, two saponification procedures were evolved—one for acid-demethylated pectins and another for all other types. The procedures differ in the amount of alkali used and the time required for saponification.

Method A (for all types of pectin except acid-deesterified). Two grams of pectin are weighed into a 600-ml. beaker, moistened with alcohol, and dissolved in 400 ml. of distilled water by agitating with a mechanical stirrer. The solution is titrated to pH 7.5 by adding 0.500 N sodium hydroxide from a 10-ml. buret. A pH meter equipped with extension electrodes is especially convenient for the titration. (The correct pH value can be obtained only on a solution which is at rest. Stirring causes the apparent pH value to drift from 0.10 to 0.50 pH unit below the correct value, depending on the ion concentration of the solution.) The neutralized solution is transferred to a 500-ml. Erlenmeyer flask, and the temperature is adjusted to 25° $\pm$ 1° C. An amount, x, of 0.500 N sodium hydroxide is added which is 5.0 ml. ($\pm$0.5 ml.) in excess of the amount equivalent to the methoxyl content of the sample. It may be necessary to run a preliminary saponification to determine the approximate amount. The solution is allowed to stand at room temperature for 30 minutes ($\pm$2 minutes). Then as much 0.500 N sulfuric acid is added as is equivalent to the amount, x, of sodium hydroxide used in the saponification, and the solution is titrated to pH 7.5 with 0.500 N sodium hydroxide. The amount of 0.500 N sodium hydroxide used in the final titration, y, subtracted from the amount added for the saponification, x, should equal 5.0 $\pm$ 0.5 ml. If not, the saponification should be repeated with a different amount of 0.500 N sodium hydroxide determined by a new estimate. The per cent methoxyl is calculated from the amount of 0.5 N sodium hydroxide, y, consumed in the saponification (after correcting for the blank):

$$\% \ CH_3O = \frac{\text{ml. of } 0.500\ N\ \text{NaOH} \times 1.55}{\text{grams of pectin}}$$

Method B (for acid-deesterified pectins). The saponification is carried out as described in Method A, except that 40.0 ml. of 0.500 N sodium hydroxide are used for the saponification, and the time is increased to 40 minutes. The final titration and the calculations are made in the same way.

Conditions of time, temperature, and alkali concentration must be adhered to rigidly if accurate and reproducible results are to be obtained. For example, in Method A an excess of 6 ml. of 0.500 N sodium hydroxide instead of 5 ml. increased the methoxyl value by 0.17%, and a 40-minute instead of a 30-minute interval increased the results by 0.25%.

Titration with a glass electrode pH meter is accurate and sensitive to additions of less than 0.01 ml. of 0.500 N sodium hydroxide. The titrations may also be carried out with an indicator, such as Hinton's (*9*), having a color change near pH 7.5. However, the sensitivity of the end point is materially reduced, and this in turn affects the precision of the method.

The methyl ester contents of a number of pectins were determined by the Myers-Baker saponification method and by the two improved procedures described above (Table II). For purposes of comparison, the results are given on a moisture-, ash-, and ethanol-free basis. In actual practice, the saponification and the pectase methoxyl values would not be determined on an ethanol-free basis, because the use of extra time for removal of adsorbed ethanol would be impractical in a rapid method of analysis. The error involved is due to the inclusion of 1 to 4% of ethanol in the weight of the sample. For many purposes an error of this magnitude is permissible. When compared with the Zeisel values, the Myers-Baker values for all except acid-demethylated pectins are from 0.35 to 0.87% (average, 0.50%) too high (Table II). The results by the two improved saponification procedures show deviations from the Zeisel values of 0.10 to 0.54% (average, 0.21%) and 0.01 to 0.17% (average, 0.10%), respectively.

The saponification method is limited, in that it attempts to balance the incomplete saponification of the methyl ester groups against the saponification error caused by the reaction of the alkali with the ballast materials or impurities of the sample. The method is rapid, but to select the correct procedure it is necessary to know the type of pectin being analyzed.

PECTASE METHOD

A reliable and convenient enzymic procedure, in which pectase is employed for the quantitative determination of pectin methyl ester, has been in use in this laboratory for the past 3 years. This method, which has not been previously reported in the literature, represents a somewhat different approach to the problem of determining pectin methyl ester. It avoids the sources of error encountered in the saponification methods and also avoids special treatment of the sample, which is often necessary for correct Zeisel results.

Tomatoes are especially rich in pectase (*13*), and the enzyme may be conveniently prepared from this source by the following procedure:

Firm ripe tomatoes are ground to a pulp in a food chopper. The acidity of the pulp is adjusted to pH 7.5 by adding 2 N sodium hydroxide with stirring, and the mixture is allowed to stand at room temperature for a few minutes, in order to free the pectase from the pulp. The extract is drained through cheesecloth and then filtered through coarse filter paper. The filtrate is covered with xylene or other suitable preservative and allowed to stand at room temperature for one day to hydrolyze any pectin present, after which it should be stored at a temperature near 0° C.

The extract is very stable and may be kept for several days at room temperature or for several months at a lower temperature—

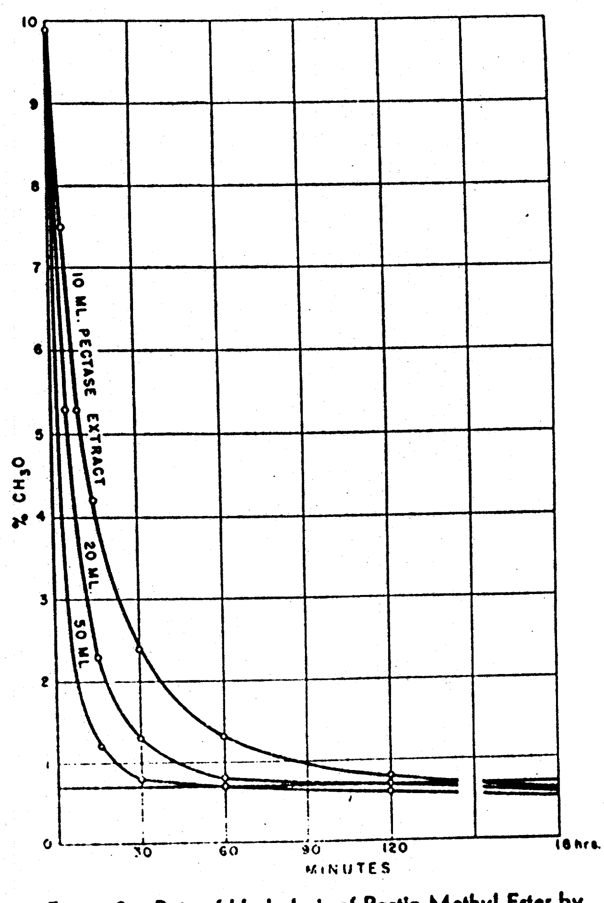

Figure 2. Rate of Hydrolysis of Pectin Methyl Ester by Pectase

Hydrolysis of 2 grams of pectin by different quantities of tomato pectase extract at 25° C. in buffered solution. Initial pH 7.5. Final pH 5.4

example, a tomato pectase extract which had been stored for months at 0° C. retained 71% of its original pectase activity.

The rate of hydrolysis of pectin methyl ester by three concentrations of pectase is shown in Figure 2. The hydrolysis proceeded rapidly but did not reach completion. After 2 hours the pectin contained from 0.65 to 0.85% methoxyl, and at 18 hours from 0.55 to 0.65%. A 3-day hydrolysis with 20 ml. of pectase extract reduced the methoxyl value to 0.50%, and a 20-day hydrolysis did not reduce it further.

A joint contribution from this laboratory and the Delaware Agricultural Experiment Station by Hills, White, and Baker (*8*) reported that tomato pectase hydrolyzed pectin to a residual methoxyl value of 1.8%. This high value was obtained by analyzing the dried pectinate by the Myers-Baker saponification method, which, as now known, gives erroneous results. The residual methoxyl was subsequently redetermined by means of the Zeisel method (on ethanol-free samples), and values were obtained in the range of 0.43 to 0.50% methoxyl.

The rate curves shown in Figure 2 suggest that an enzyme method can be devised which yields methoxyl values in agreement with the Zeisel procedure, provided a correction is made for residual unhydrolyzed methyl ester. The method adopted uses 20 ml. of tomato pectase extract and a reaction time of 2 hours at 25° C. For most pectins the correction to be applied for the residual methyl ester is 0.75% methoxyl. This value for the residual methyl ester was determined by Zeisel analysis of the dried residues of six pectin samples which had been subjected to pectase hydrolysis as described above. The samples studied represent a wide range of types of pectin and are designated in Table II as Nos. 123, 102, 115, 91C, 91H, and 115B. Their respective residual methoxyl values were 0.64, 0.63, 0.75, 0.84, 0.85, and 0.80%; average 0.75 ± 0.09% methoxyl.

For acid-deesterified pectins analyzing less than 3% methoxyl a smaller correction is necessary, because extensive acid treatment hydrolyzes a portion of the methyl ester resistant to pectase hydrolysis. Determination of the residual methoxyl values for a series of acid-deesterified pectins containing 0.6 to 3.0% methoxyl showed that the correction to be added for such pectins is 25% of the observed pectase value.

The recommended procedure is as follows:

Two grams of pectin are weighed into a 600-ml. beaker, moistened with ethanol, and dissolved with 400 ml. of distilled water with the aid of a mechanical stirrer. Ten milliliters of 2 *N* sodium acetate solution and 10 ml. of 2% sodium oxalate solution are added. The pectin solution is neutralized to pH 7.50 by adding 0.500 *N* sodium hydroxide from a 10-ml. buret, a pH meter equipped with extension leads being used to determine the exact end point. Twenty milliliters of tomato pectase extract (adjusted to pH 7.50 before use) are added to the neutralized pectin solution. The contents of the beaker are mixed by stirring and allowed to stand at room temperature for 2 hours. At the end of this time the solution is titrated to pH 7.50. A blank determination is made without the pectin, and the indicated correction is applied. The methyl ester content of the sample, calculated as per cent methoxyl, is

$$\% \text{ CH}_3\text{O} = \frac{\text{ml. of 0.500 } N \text{ NaOH} \times 1.55}{\text{grams of pectin}} + 0.75$$

If the sample is an acid-deesterified pectin with a methoxyl value less than 3%, the formula is

$$\% \text{ CH}_3\text{O} = \frac{\text{ml. of 0.500 } N \text{ NaOH} \times 1.94}{\text{grams of pectin}}$$

Results obtained by the pectase method are affected only slightly by variations in the conditions of hydrolysis. For example, as shown in Figure 2, varying the added pectase from 50 to 250% of the stipulated amount changed the residual methoxyl by only 0.10%. The pectase activity of a large number of tomato extracts prepared in the manner here described showed variations of less than 20% from the average. Therefore it is not necessary to standardize the amount of pectase used in the hydrolysis, because normal variations in activity from one preparation to another would not be sufficient appreciably to affect accuracy.

The methoxyl values for a number of pectins analyzed by the pectase method are recorded in the last column of Table II. The analyses show an average deviation from the Zeisel values of 0.09%. The over-all precision, as determined by agreement between duplicate analyses made on different days and with different pectase extracts, was 0.02% methoxyl. The pectase method gives a satisfactory degree of accuracy for all types of pectin. For more rapid determinations of methyl ester, as may be required in plant control work, the 2-hour pectase method may be reduced to 30 minutes by using 50 ml. of tomato pectase extract (see Figure 2). The value for the residual methyl ester in this case is 0.85% methoxyl.

SUMMARY

The Zeisel and saponification methods for determining the methyl ester content of pectic substances have been studied, sources of error indicated, and remedial measures suggested. A third method, employing the enzyme pectase, has been developed.

The presence of adsorbed ethanol in pectin was confirmed by the detection of ethyl iodide in the Zeisel distillate. This source of error was eliminated by an improved water-vapor treatment of the pectin sample.

The published saponification methods usually give high values, owing to the action of alkali on the non galacturonide constituents of pectin. The nature and amount of these interfering substances in different types of pectins vary widely. In order to analyze all types, it was necessary to develop two saponification methods. For acid-demethylated pectins the Myers-Baker method was modified by using a shorter saponification time. For other types of pectins, which contain appreciable amounts of interfering substances, a saponification procedure was developed which gives more nearly correct results than the methods commonly used.

A new procedure is presented which utilizes the specific action of the enzyme pectase for hydrolyzing pectin methyl ester, with a correction for the methyl ester remaining unhydrolyzed.

With pectin samples free of interfering solvent the Zeisel method gives accurate results and is considered to be the reference method. However, it is time-consuming, requires special apparatus, and necessitates a pretreatment of the sample. The saponification methods presented are rapid, but presuppose a knowledge of the type of pectin being analyzed. The pectase method is preferred for general use; it is more rapid than the Zeisel method, involves fewer sources of error than the saponification method.

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